The Plant Cell, Vol. 11, 865–874, May 1999, www.plantcell.org © 1999 American Society of Plant Physiologists

CHL1 Is a Dual-Affinity Nitrate Transporter of Arabidopsis Involved in Multiple Phases of Nitrate Uptake

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Higher plants have both high- and low-affinity nitrate uptake systems. These systems are generally thought to be genetically distinct. Here, we demonstrate that a well-known low-affinity nitrate uptake mutant of Arabidopsis, *chl1*, is also defective in high-affinity nitrate uptake. Two to 3 hr after nitrate induction, uptake activities of various *chl1* mutants at 250 μM nitrate (a high-affinity concentration) were only 18 to 30% of those of wild-type plants. In these mutants, both the inducible phase and the constitutive phase of high-affinity nitrate uptake activities were reduced, with the inducible phase being severely reduced. Expressing a *CHL1* cDNA driven by the cauliflower mosaic virus 35S promoter in a transgenic *chl1* plant effectively recovered the defect in high-affinity uptake for the constitutive phase but not for the induced phase, which is consistent with the constitutive level of *CHL1* expression in the transgenic plant. Kinetic analysis of nitrate uptake by *CHL1*-injected Xenopus oocytes displayed a biphasic pattern with a Michaelis–Menten K_m value of ~50 μM for the high-affinity phase and ~4 mM for the low-affinity phase. These results indicate that in addition to being a low-affinity nitrate transporter, as previously recognized, CHL1 is also involved in both the inducible and constitutive phases of high-affinity nitrate uptake in Arabidopsis.

INTRODUCTION

Nitrate uptake is a physiological process critical for plant growth. Plants have evolved a host of transport systems to accommodate external nitrate concentration levels that can vary up to 10,000 times (Jackson and Caldwell, 1993), and expression of these systems appears to be tightly regulated (Hoff et al., 1994; Crawford, 1995; Glass and Siddiqi, 1995; Wirén et al., 1997). Early experiments with kinetic measurements have identified at least three nitrate transport systems in higher plants. One operates at high nitrate concentrations (over ~0.5 mM) and is constitutively expressed. It is usually referred to as the cLATS (for constitutive low-affinity transport system); at ~0.5 mM nitrate, uptake is performed by two HATS (for high-affinity transport system). One is constitutive (cHATS) and the other is inducible (iHATS) (reviewed in, e.g., Larsson and Ingemarsson, 1989; Glass and Siddiqi, 1995). Extensive efforts have been directed in recent years toward cloning and characterizing the genes that are responsible for these transport systems (reviewed in Crawford and Glass, 1998; Daniel-Vedele et al., 1998). As a result, our knowledge about nitrate transporters of higher plants is rapidly accumulating, and it is now clear that the three-system (cLATS, iHATS, and cHATS) model derived from early physiological studies is overly simplified.

For example, cloning and functional characterization of the Arabidopsis nitrate transporter gene *CHL1* (*AtNRT1*) (Tsay et al., 1993; Huang et al., 1996) have shown that *CHL1* is responsible for an additional LATS that is nitrate inducible, that is, iLATS, which was not revealed by previous physiological characterizations of nitrate uptake. This finding necessitates a bipartite model for low-affinity nitrate uptake of Arabidopsis (i.e., *CHL1* for the iLATS and an unknown gene for the cLATS; Huang et al., 1996). The existence of the iLATS in higher plants may in fact be general. A *CHL1* homolog from Brassica, *BnNRT1;2*, is nitrate inducible and displays low-affinity nitrate activities in Xenopus oocytes (Zhou et al., 1998). In additiion, two *CHL1* homologs identified from tomato, *LeNRT1-2* and *LeNRT1-1*, are nitrate-induced and constitutively expressed, respectively (Lauter et al., 1996).

For the HATS in higher plants, several genes from a second nitrate transporter family called *NRT2* have been identified. They are homologous to the high-affinity nitrate transporter gene *crnA* of *Aspergillus nidulans* (Unkles et al., 1991) and *NAR3/NAR4* of Chlamydomonas (Quesada et al., 1994). They include *BCH1* and *BCH2* from barley (Trueman et al., 1996), *NpNRT2-1* from tobacco (Quesada et al., 1997; Krapp et al., 1998), and *AtNRT2;1* and *AtNRT2;2* from Arabidopsis (Crawford and Glass, 1998; Daniel-Vedele et al., 1998; Filleur and Daniel-Vedele, 1999). All of these *NRT2* genes are expressed in roots, and their expression is nitrate inducible. Together, these recent findings seem to support the notion that *NRT2* genes constitute iHATS, whereas *NRT1* genes constitute the LATS.

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We now report a surprising discovery that CHL1 is also directly involved in high-affinity nitrate uptake. Presented herein are results from several in vivo (in plant) and in vitro (in Xenopus oocyte) uptake assays that all support this conclusion. These assays provide convincing molecular evidence to indicate that a single nitrate transporter protein can be responsible for more than one mode of the distinct uptake activities observed in kinetic studies. Possible reasons as to why the high-affinity nitrate uptake function of CHL1 has not been revealed in previous studies are discussed.

RESULTS

Reduced High-Affinity Nitrate Uptake in the *chl1-5* Mutant

Because nitrate uptake at 250 μM is contributed primarily by the HATS (Doddema and Telkamp, 1979; Meharg and Blatt, 1995), wild-type and *chl1-5* plants were grown with 12.5 mM $(NH_4)_2$ succinate as the sole nitrogen source for 12 days and then transferred to a nitrate medium of 250 μM KNO_3 at pH 5.5 for uptake assays. As shown in Figure 1A, two phases of nitrate uptake, a constitutive phase and an induced phase occurring ~1 hr after the transfer, were observed in the wild-type plant. In comparison, the uptake activities of *chl1-5* mutant were significantly reduced at all time points. This defect in high-affinity nitrate uptake of *chl1-5* was also evident when the assays were performed at pH 7.0 (Figure 1B). The uptake rate of the wild-type plant under this condition was linear and showed no induced phase.

It has been reported that the cytoplasmic pH of a *chl1* mutant (*chl1-1*) is more acidic than that of the wild type when plants are grown in ammonia (nitrate-free) media (Meraviglia et al., 1996) but more alkaline when grown in NH_4NO_3 (Romanni et al., 1996). Because nitrate uptake systems of Arabidopsis use a proton-coupled electrogenic scheme for transport activity (Meharg and Blatt, 1995), a reduced pH gradient across the plasma membrane in a nitrate-free medium might cause the observed defect. To address this possibility, *chl1-5* and wild-type plants were grown under the same growth conditions described above, except that the nitrogen source, $(NH_4)_2$ succinate, was replaced by NH_4NO_3. As shown in Figure 1C, under this growth condition, the high-affinity nitrate uptake activity *chl1-5* was also significantly reduced from that of the wild type, indicating that the uptake defect is associated with *chl1-5* and not a consequence of differences in cytoplasmic pH.

Chlorate Sensitivity Test

Chlorate is a toxic nitrate analog that has been used to screen for mutants defective in nitrate uptake or reduction

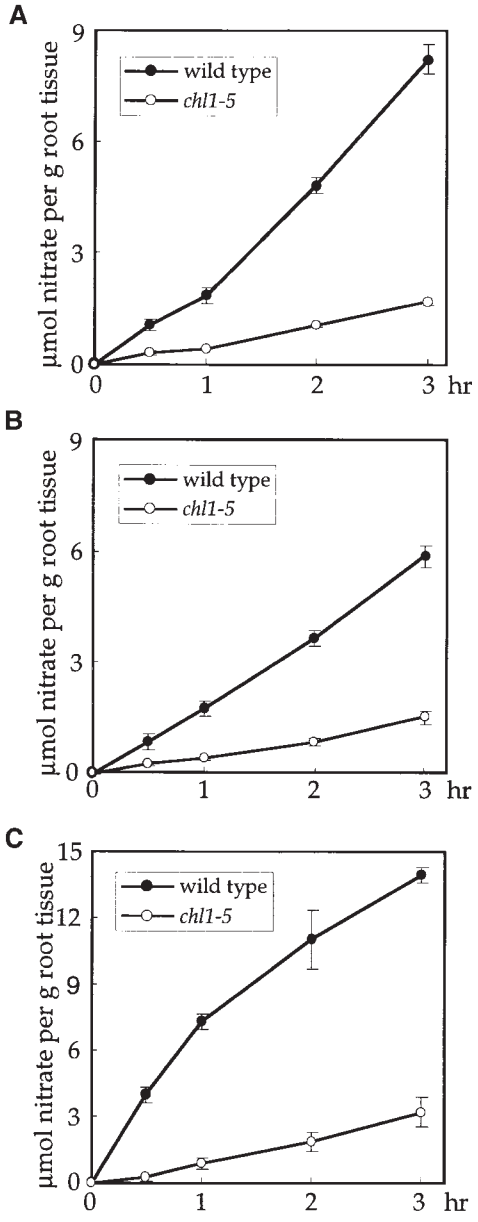

Figure 1. Time Course of Nitrate Uptake by Arabidopsis Wild-Type and *chl1-5* Plants.

(A) Nitrate uptake studies of ammonium-grown plants at pH 5.5.
(B) Nitrate uptake studies of ammonium-grown plants at pH 7.0.
(C) Nitrate uptake studies of NH_4NO_3-grown plants at pH 5.5.
Seedlings were grown in a medium containing 12.5 mM $(NH_4)_2$ succinate (**[A]** and **[B]**) or NH_4NO_3 **(C)** for 12 days. Plants were then washed and resuspended into a medium with 250 μM KNO_3 at pH 5.5 (**[A]** and **[C]**) or pH 7.0 **(B)** for uptake assays. The amount of nitrate depleted from the medium was monitored at the time points indicated.

(reviewed in Warner and Kleinhofs, 1992; Crawford and Arst, 1993; Hoff et al., 1994; Crawford, 1995). If *chl1-5*, a chlorate-resistant mutant in the low-affinity range of concentration (Tsay et al., 1993), is also defective in high-affinity nitrate uptake, it should confer chlorate resistance in the high-affinity range as well. As illustrated in Figure 2, when plants were treated with 10 μM chlorate along with 50 μM nitrate, severe chlorosis was observed in the wild-type plant, but the *chl1-5* mutant was resistant to the same treatment. The average shoot fresh weight of 30 15-day-old plants treated with 10 μM chlorate was 1.4 ± 0.2 mg for the wild-type plants and 7.4 ± 0.6 mg for *chl1-5* plants.

High-Affinity Nitrate Uptake of Other *chl1* Mutants

chl1-5, generated by γ-irradiation (Tsay et al., 1993), is a deletion mutant, but the size of the deletion has not been determined. Therefore, we could not rule out the possibility that sequence deletion outside of *CHL1* itself caused the observed defect of *chl1-5*. To clarify this issue, we isolated three additional *chl1* mutants, *chl1-7*, *chl1-8*, and *chl1-9*, from ethyl methanesulfonate (EMS)–mutagenized pools. The three EMS-generated mutants, as well as *chl1-1* (Braaksma and Feenstra, 1973), were more likely to have point mutations or small deletions because of their ability to express *CHL1* mRNA (Figure 3A). This is in contrast to the observation of virtually no detectable *CHL1* mRNA in *chl1-5* plants (data not shown). The larger *CHL1* mRNA in the *chl1-1* mutant (Figure 3A) might result from, for example, a mutation around the splicing site, the transcription initiation site, or the termination site. Although the exact nature of the defect in these mutants remains to be determined, uptake measurements with 250 μM nitrate, as shown in Figure 3B, indicate that like *chl1-5*, all of these mutants are impaired in high-affinity nitrate uptake. As expected from their screening conditions (see Methods), uptake studies with 8 mM nitrate showed that these mutants are also all defective in low-affinity nitrate uptake (data not shown).

Figure 2. Chlorate Sensitivity Test for Arabidopsis Wild-Type and *chl1-5* Plants.

Six Arabidopsis wild-type plants (top) and six *chl1-5* plants (bottom) were treated with chlorate. Photographs were taken of 14-day-old plants grown with 2.5 mM $(NH_4)_2SO_4$ and 50 μM KNO_3. Plants were treated with 10 μM chlorate before vernalization and again 3, 6, and 9 days after germination.

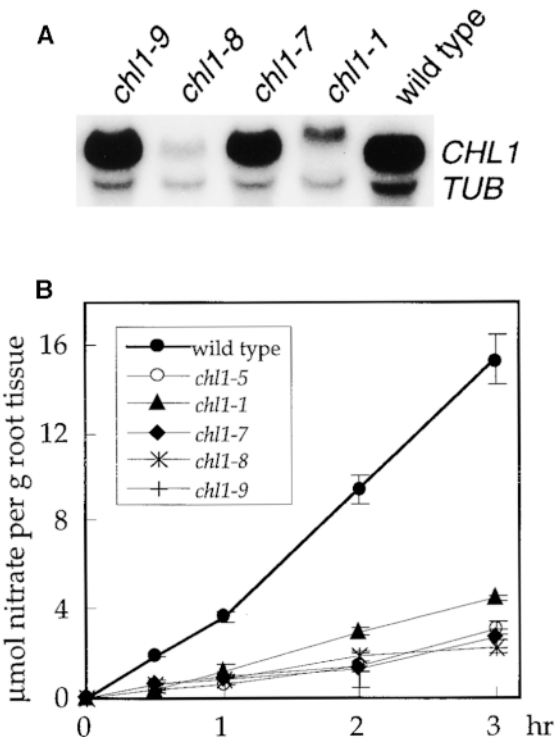

Figure 3. High-Affinity Nitrate Uptake Defect in Various Arabidopsis *chl1* Plants.

(A) RNA gel blot analysis of various *chl1* mutants. Total RNA (10 μg) was isolated from the root tissues of plants grown vertically on an ammonium plate without nitrate and then shifted to a medium of 25 mM KNO_3 for 2 hr. RNA transferred to a Hybond N membrane was hybridized with ^{32}P-radiolabeled DNA from *CHL1* and the Arabidopsis tubulin gene (*TUB*) as an internal control (Marks et al., 1987).
(B) Nitrate uptake activities of various *chl1* mutants. Seedlings were grown in a medium containing 12.5 mM $(NH_4)_2$ succinate for 12 days. Plants were then washed and resuspended into a medium with 250 μM KNO_3 at pH 5.5 for the uptake assay. The amount of nitrate depleted from the medium was monitored at the time points indicated.

Recovery of the Uptake Defect by 35S–*CHL1*

To further verify that the defect in high-affinity nitrate uptake is associated with *CHL1*, we introduced cauliflower mosaic virus 35S–*CHL1* cDNA into the deletion mutant *chl1-5.* As shown in Figure 4A, the high-affinity uptake reduced in the deletion mutant was effectively recovered in the transgenic plants expressing 35S–*CHL1*, although in the induced phase, the uptake rate was not as high as that of the wild type. This rate difference in the induced uptake correlates with the expression patterns of *CHL1* in these plants. Before nitrate induction, the *CHL1* mRNA level in 35S–*CHL1* plants was approximately the same as (or slightly higher than) that of the wild-type plants. After exposure to 250 μM nitrate, *CHL1* expression started to increase at 0.5 hr and peaked at 2 hr in wild-type plants but remained unchanged in 35S–*CHL1* plants. That is, nitrate-induced *CHL1* expression was absent in 35S–*CHL1* plants (Figures 4B and 4C). The expression data indicated that in these experiments, the 35S promoter introduced into the *chl1-5* deletion background was not as strong as the native promoter of *CHL* during nitrate induction.

It is of interest that there is a good temporal correlation between the increase of high-affinity nitrate uptake activity and the *CHL1* mRNA level in wild-type plants, because both of them increased significantly 1 hr after induction (Figures 4A and 4B). In comparison, the quantitative correlation is poor: whereas the mRNA level increased sixfold to approximately sevenfold after induction, CHL1-specific activity, obtained by subtracting the activity of the *chl1-5* mutant from that of the wild type, increased only 1.4-fold to ~1.8-fold. The poor correlation may indicate that not all of the *CHL1* mRNA expressed was translated to function for high-affinity uptake, and post-transcriptional regulation or protein modification might thus be required for most of the observed high-affinity uptake activities attributable to CHL1. Because little is known about how a nitrate transporter is regulated at the protein level, this is a hypothesis worth further examination.

Nitrate Uptake Assay in Xenopus Oocytes

The suggestion that the high-affinity phase of nitrate uptake is an intrinsic function of CHL1 as opposed to being the consequence of another protein being regulated or activated by CHL1 can be further supported by uptake measurements using the Xenopus oocyte system. However, unlike the low-affinity phase of uptake (Tsay et al., 1993), the high-affinity phase of uptake elicits an electrical response too small (~10 nA) to be easily distinguishable from the background noise of the oocyte system. Taking this into consideration, we investigated here the amount of nitrate taken up by *CHL1*-injected oocytes. For the purpose of comparison, HPLC analysis for both high-affinity and low-affinity nitrate uptake was performed. However, due to the

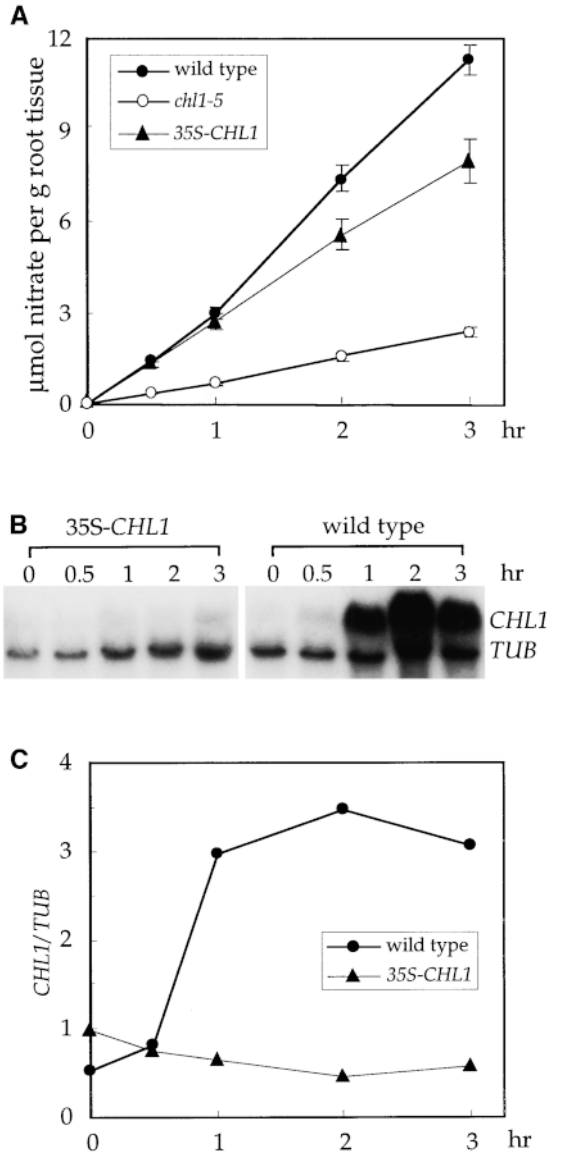

Figure 4. Recovery of the Reduced High-Affinity Nitrate Uptake by 35S–*CHL1* Plants.

(A) Time course of nitrate uptake activity in wild-type, *chl1-5* deletion-mutant, and transgenic *chl1-5* plants containing 35S–*CHL1*. See Figure 3B legend for seedling growth and uptake measurements.

(B) RNA gel blot analysis of *CHL1* mRNA levels in wild-type and transgenic *chl1-5* plants containing 35S–*CHL1*. The mRNA levels of plants grown under the same conditions for uptake measurements in **(A)** were determined by RNA gel blot analysis at the time points indicated. The blot was hybridized with ^{32}P-radiolabeled *CHL1* cDNA as well as a ^{32}P-radiolabeled Arabidopsis tubulin gene probe (*TUB*) to verify sample loading (Marks et al., 1987; Tsay et al., 1993).

(C) Quantification of the *CHL1* mRNA levels in response to nitrate induction of 250 μM. Data are based on densitometer measurements of the hybridizing bands in **(B)**. *CHL1* mRNA level was normalized with respect to the level of tubulin (*TUB*) mRNA.

limitation of the sensitivity of HPLC nitrate detection, different procedures were performed for the two phases. For the low-affinity uptake measurements, groups of five oocytes were incubated with 10 mM nitrate at pH 5.5 for 3 hr and then washed and assayed for the nitrate retained within the oocyte. For the high-affinity uptake measurements, oocytes from the same batch of oocytes used for the low-affinity uptake assay were incubated with 250 μM nitrate at pH 5.5 for 3 hr, and the amount of nitrate depleted from the external solution was determined. For these measurements, a significant increase in both low-affinity (Figure 5A) and high-affinity (Figure 5B) nitrate uptake was detected in *CHL1*-injected oocytes.

To determine whether dual-affinity nitrate uptake is also a property of other CHL1 homologs, the same experiments were repeated for a newly isolated Arabidopsis *CHL1* homolog, *NTL1* (expressed sequence tag clone YAP049; GenBank accession number AF073361), which shares 36% amino acid sequence identity with CHL1. As shown in Figures 5A and 5B, only low-affinity but not high-affinity nitrate uptake activity was observed in *NTL1*-injected oocytes. Similar results were obtained with oocytes isolated from four different donor frogs for *CHL1* and two for *NTL1*. Thus, the ability to operate at both high- and low-affinity concentrations appears to be specific to CHL1. One might argue that the high-affinity uptake of CHL1 observed is a carryover of its low-affinity uptake activity, but the lack of high-affinity uptake in *NTL1*-injected oocytes and K_m analysis (see below) argue against this possibility.

The activity of high-affinity nitrate uptake observed in *CHL1*-injected oocytes is pH dependent. As presented in Figure 5C, the uptake of 250 μM nitrate at pH 5.5 was 2.8 times higher than that at pH 7.4. This pH dependency is consistent with the suggestion that high-affinity nitrate uptake of Arabidopsis is mediated by a proton-coupled transporter (Meharg and Blatt, 1995).

These oocyte data, in conjunction with the in vivo uptake measurements described above, suggest that CHL1 itself is functional in both the low- and high-affinity phases. To further evaluate this suggestion, we performed kinetic analysis of nitrate uptake by *CHL1*-injected oocytes under 11 different concentrations of nitrate covering both phases (from 16 μM to 30 mM). The amount of nitrate taken up was linear against the time of incubation over the experimental period (i.e., showing no saturation of the uptake capacity; data not shown), whereas the nitrate uptake rate was concentration dependent, revealing a biphasic pattern (Figure 6A). The high-affinity phase appeared saturated at 65 to 180 μM, whereas the low-affinity phase appeared to approach saturation between 10 and 30 mM (Figure 6A). Similar results were obtained with three different batches of oocytes isolated from different frogs (Table 1).

The average K_m values obtained by fitting these measurements to the Michaelis–Menten equation were 49 ± 7 μM for the high-affinity phase and 4.0 ± 1.2 mM for the low-affinity phase (Table 1). The low-affinity K_m determined here

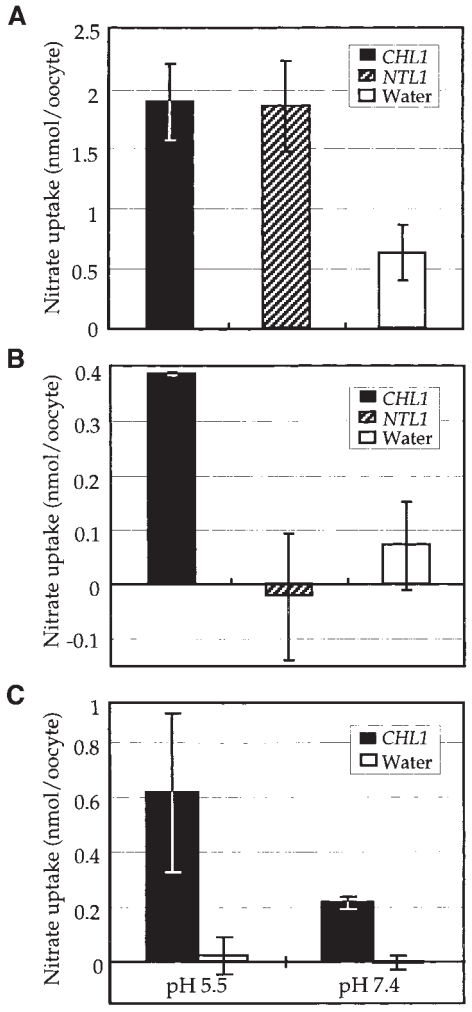

Figure 5. Nitrate Uptake Activities in *CHL1*-, *NTL1*-, and Water-Injected Xenopus Oocytes.

(A) Low-affinity nitrate uptake activities determined with 10 mM NO_3^-. Oocytes were incubated with 10 mM NO_3^-, pH 5.5, for 3 hr and then assayed for retained nitrate by using HPLC.

(B) High-affinity nitrate uptake activities determined with 250 μM NO_3^-. Oocytes were incubated with 250 μM NO_3^-, pH 5.5, for 3 hr, and the amount of nitrate depleted from the medium was then determined by HPLC.

(C) pH dependence of CHL1 activity in high-affinity nitrate uptake. Oocytes were incubated with 250 μM NO_3^-, pH 5.5 or pH 7.4, for 3 hr, and the amount of nitrate depleted from the solution was then determined.

Every data point for **(A)** to **(C)** represents the average value obtained from HPLC measurements of two or three batches, each consisting of five oocytes. Error bars indicate standard deviations.

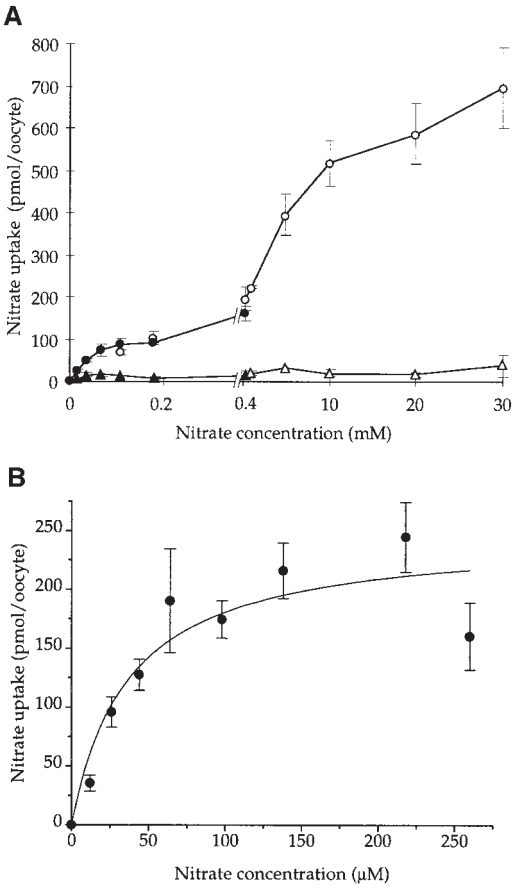

Figure 6. Kinetic Analysis of Nitrate Uptake in *CHL1*-Injected Oocytes.

(A) Uptake studies of *CHL1*-injected (circles) and water-injected (triangles) oocytes with nitrate concentrations from 16 μM to 30 mM. Oocytes were incubated with nitrate medium at pH 5.5 for 3 hr. Open symbols represent nitrate uptake determined by the amount of nitrate retained in the *CHL1*-injected oocytes, and closed symbols represent nitrate uptake determined by the amount of nitrate depleted from the solution (see Methods). At concentrations of 110, 180, and 380 μM, measurements of both retained nitrate and depleted nitrate were taken. The scale from 0 to 0.4 mM was enlarged. Every data point is the average value obtained from three duplicate experiments, each consisting of five oocytes.

(B) A more detailed measurement in the high-affinity range of concentration. Nitrate uptaken was determined as the amount of nitrate depleted from the solution. Every data point is the average value obtained from three duplicate experiments, each consisting of five oocytes. The curve was obtained by fitting to the Michaelis–Menten equation by using a nonlinear least-squares method in the Program Original 5.0 (Microcal Software, Inc., Northampton, MA).

by HPLC analysis is comparable with that (8.5 ± 3.1 mM) derived from nitrate-elicited currents previously reported (Huang et al., 1996). Also shown in Figure 6A, the two methods of HPLC analysis used for measuring nitrate uptake of the two phases, respectively, gave comparable values for three overlapping concentration points. A more detailed analysis of the high-affinity phase, shown in Figure 6B, further illustrated a clear saturation of the uptake rate in this phase, even though different V_{max} values (cf. Figures 6A and 6B; see also Table 1) presumably resulted from different levels of expression in oocytes from different frogs.

DISCUSSION

Significantly reduced activities in high-affinity nitrate uptake were observed for various *chl1* mutants (Figure 3B), despite the fact that these mutants were selected for their deficiency in low-affinity nitrate uptake. This observation was corroborated by results from a chlorosis test (Figure 2), by the fact that a transgenic plant expressing 35S–*CHL1* cDNA in the deletion background recovered much of the reduced uptake (Figure 4A), and by uptake assays using Xenopus oocytes (Figures 5 and 6). Kinetic analysis of nitrate uptake activity in *CHL1*-injected oocytes yielded two saturable phases, one in the micromolar range and the other in the millimolar range. The K_m of ~50 μM determined for the high-affinity phase is comparable with the values of 23 to 44 μM deduced from in-the-plant kinetic measurements of the Arabidopsis HATS (Doddema and Telkamp, 1979; Meharg and Blatt, 1995; Touraine and Glass, 1997). Together, these data indicate that in addition to inducible low-affinity uptake (Huang et al., 1996), CHL1 is directly involved in high-affinity uptake of nitrate.

The activities in both the constitutive phase (within ~1 hr of the shift to the nitrate medium) and the inducible phase of high-affinity nitrate uptake were reduced in the *chl1* mutants (Figures 1A, 3B, and 4A), indicating that CHL1 contributes to the uptake function of both iHATS and cHATS. These data may be interpreted by two differing uptake mechanisms: (1) the basal level of *CHL1* expression accounted for some of the activities of cLATS (i.e., a "genetic leak" mechanism; Behl et al., 1988); or (2) less nitrate was taken up during the constitutive phase to induce the inducible phase, thereby resulting in a substantial reduction of iHATS in these *chl1* mutants. We believe the second mechanism is less likely for three reasons. First, iHATS activities of the *chl8* mutant are wild-type compatible, even though *chl8* cHATS activities are severely suppressed at pH 7.0 (Wang and Crawford, 1996). Therefore, a defect in the constitutive phase will not necessarily cause a defect in the inducible phase. Second, *CHL1* was easily induced by nitrate (in the micromolar range) in the wild-type plants, and the induced expression correlated temporally with the induced high-affinity nitrate uptake activity (Figure 4). Third, consistent with the constitutive expression level (Figure 4C), nearly all of the cHATS but little of the iHATS activities were recovered in the 35S–*CHL1* plants (Figure 4A and Table 2), indicating that nitrate-induced *CHL1* expression was required for at least part of the iHATS activities observed in the wild-type plants.

Although the uptake reduction in the inducible phase ap-

Table 1. Kinetic Parameters of Nitrate Uptake Measured in *CHL1* Complementary RNA–Injected Oocytes

	High-Affinity (HA) Phase[b]		Low-Affinity (LA) Phase[b]		
Experiment No.[a]	K_m^{HA} (μM)	V_{max}^{HA} (pmol/Oocyte)	K_m^{LA} (mM)	V_{max}^{LA} (pmol/Oocyte)	$V_{max}^{LA}/V_{max}^{HA}$
1	50 ± 9	123 ± 8	5.1 ± 1.0	783 ± 44	6.4
2	56 ± 34	114 ± 23	4.6 ± 3.0	828 ± 150	7.3
3	51 ± 9	163 ± 9	2.4 ± 0.4	1046 ± 37	6.4
4	37 ± 17	247 ± 33	—[c]	—	—
Average	49 ± 7		4.0 ± 1.2		6.7 ± 0.4

[a]Experiments 1, 2, 3, and 4 were performed with oocytes from four different frogs. The data from experiments 1 and 4 were used to produce Figure 6A and 6B, respectively, by plotting the amounts of nitrate taken up during the 3-hr incubation time as a function of external nitrate concentration.

[b]K_m and V_{max} were obtained by fitting to the Michaelis–Menten equation, using a nonlinear least-squares method of Program Original 5.0 (Microcal Software, Inc.).

[c]—, not determined

peared to be severe in these *chl1* mutants, a small increase in uptake activity after nitrate induction was nevertheless detectable by a careful examination of the data (e.g., see Table 2 for the *chl1-5* mutant). This observation and that of the partially defective cHATS in *chl1* mutants implicate activities of other cHATS and iHATS genes in these uptake experiments. This suggestion is in line with the properties of *chl8* (Wang and Crawford, 1996) and of the *NRT2* genes recently identified in Arabidopsis and other higher plants (Trueman et al., 1996; Quesada et al., 1997; Krapp et al., 1998; also reviewed in Wirén et al., 1997; Crawford and Glass, 1998; Daniel-Vedele et al., 1998; Filleur and Daniel-Vedele, 1999). In addition, because nitrate uptake activities are modulated by growth conditions (discussed below), growth conditions different from those used here may result in more activities of other HATS genes.

That CHL1 is also a HATS is at odds with almost all of the previous kinetic measurements on *chl1* plants, including the classic experiment of Doddema and Telkamp (1979) and, more recently, that of Touraine and Glass (1997). One exception, however, is the reduced chlorate uptake at both high- and low-affinity ranges of concentration in a *chl1* mutant recorded by Scholten and Feenstra (1986; also see the review by Larsson and Ingemarsson, 1989). Therefore, one must ask why CHL1 has been taken to function only as a LATS for such a long time. We believe the answer lies in the complex behavior of *CHL1*, which can easily be affected by growth conditions and other environmental factors such as pH. For example, the induced phase of HATS in wild-type plants was altered when pH of the nitrate medium was 7.0 instead of 5.5 (Figures 1A and 1B).

More perplexing are the recent results of Touraine and Glass (1997): when plants were grown in KNO_3, the authors reported no difference in the $^{13}NO_3^-$ influx between the wild type and *chl1-5* in either the high-affinity (10 to 150 μM) or low-affinity (1 to 10 mM) range of nitrate concentration; in contrast, when the growth condition was changed and plants were grown in NH_4NO_3, the observed low-affinity $^{15}NO_3^-$ influx was approximately four times that of the mutant. The consequence of the change in growth conditions on high-affinity uptake was not addressed.

We recently showed that in mature roots, *CHL1* is primarily expressed in the cortex and endodermis (Huang et al., 1996); therefore, for trace amounts of $^{15}NO_3^-$ or $^{13}NO_3^-$ to be transported by CHL1, they must diffuse into the inner space near these cells to replace the unlabeled NO_3^-. It follows that compared with the LATS, the much lower concentration (in the micromolar range) operated by the HATS may render detecting the influx of $^{15}NO_3^-$ or $^{13}NO_3^-$ more difficult or require a longer measurement time. Also, for the oocyte experiments, it is difficult to detect the electrical response of *CHL1*-injected oocytes when the transporter is operating at its high-affinity uptake mode, because the nitrate uptake capacity of CHL1 at high-affinity phase is only ~15% of the capacity at low-affinity phase (Table 1). Consequently, compared with the 20- to 60-nA typical response of the 10 mM nitrate (Tsay et al., 1993), the elicited current for the high-affinity phase is only 3 to 10 nA. This value is not easily distinguished from the values of water-injected control oocytes.

The fact that there are multiple genes (*CHL1*, *CHL8*, and *NRT2*) required to compose the HATS of nitrate uptake in Arabidopsis suggests that, subject to regulation, these genes may interact and complement each other. It follows that the uptake phenotype of *chl1* may not always be observable because, under certain conditions, the expression of *CHL1*, an inducible gene, may be dominated by the expression of another transporter gene. On the other hand, the fact that CHL1 plays roles in multiple systems of nitrate uptake (iLATS, iHATS, and cHATS) could explain the frequent occurrence of *chl1* we observed when plants were screened with 2 mM chlorate in the presence of 2.5 mM NH_4NO_3. That is, even though seven different chlorate-resistant loci (*chl1*

Table 2. Nitrate Uptake Rate Observed in Wild-Type, *chl1-5*, and Transgenic *chl1-5* Plants Containing 35S–*CHL1*

Plants	Nitrate Uptake Rate (μmol/g/hr) Observed[a]		
	Within 30 min Exposed to 250 μM Nitrate	Within 2 to 3 hr Exposed to 250 μM Nitrate	Nitrate Uptake Rate Increased after Nitrate Induction
Wild type	2.79 ± 0.05	3.90 ± 0.10	1.11
chl1-5	0.68 ± 0.02	0.84 ± 0.05	0.16
Transgenic 35S–*CHL1*	2.68 ± 0.20	2.44 ± 0.21	−0.24

[a] Nitrate uptake rates were calculated from the time course of nitrate uptake activity shown in Figure 4A.

to *chl7*) have been identified by using the same screening conditions (reviewed in Crawford and Arst, 1993; Hoff et al., 1994), four of the five chlorate-resistant mutants that we obtained were allelic to *chl1* (data not shown). In contrast, a nitrate reductase double mutant (*nia1 nia2*) and *chl8*, but not *chl1*, were derived by treating Arabidopsis plants with 100 μM chlorate in the absence of nitrate (Wang and Crawford, 1996). These observations indicate that although chlorate is a powerful tool for screening nitrate uptake and reduction mutants, various concentrations of chlorate with nitrate in the absence or in the presence of differing concentrations lead to a spectrum of distinct mutants.

In this work, we show that CHL1, long considered to be only a component of LATS, is also a component of HATS. The dual-affinity uptake of CHL1 observed here parallels that of the Arabidopsis potassium transporter AtKUP1 that has been reported recently (Fu and Luan, 1998; Kim et al., 1998). These observations raise the question of how a dual-affinity transporter works. A single transporter may possess two substrate binding sites with different affinities, as has been suggested for *Escherichia coli lac* permease (Lolkema et al., 1991); alternatively, a transporter with a single substrate binding site may function in two states, with the transformation from one state to the other regulated by a post-translational modification mechanism such as phosphorylation. In this context, it is of interest that phosphorylation has been shown by Xenopus oocyte analysis to regulate transport activity of a seed-specific water channel (Maurel et al., 1995). Further analysis of the structural requirement and the underlying mechanism of the dual-affinity capability of CHL1 should yield insights into the complex regulation of nitrate uptake in higher plants.

METHODS

Plant Materials

Arabidopsis thaliana chl1 mutants used include the following: *chl1-5*, a deletion mutant in the Columbia ecotype background (Tsay et al., 1993); *chl1-1* (ecotype Landsberg *erecta*), isolated by Braaksma and Feenstra (1973) and characterized by Doddema et al. (1978); *chl1-7*, *chl1-8*, and *chl1-9* (ecotype Columbia), isolated by their resistance to 2 mM chlorate in the presence of 2.5 mM NH_4NO_3 from independent pools of ethyl methanesulfonate (EMS)–mutagenized M_2 seeds either purchased from Lehle Seeds Co. (Tucson, AZ) or received as a gift from J. Ecker (University of Pennsylvania, Philadelphia). Complementation tests were performed between *chl1-5* and *chl1-7*, *chl1-8*, or *chl1-9*, and the results indicate that they are allelic. The transgenic plant was constructed by introducing 35S–*CHL1* cDNA into *chl1-5* (Huang et al., 1996).

Nitrate Uptake Assay of Arabidopsis Plants

Arabidopsis seeds were surface sterilized and sown in 125-mL flasks with a 25-mL nutrient solution containing 12.5 mM $(NH_4)_2$ succinate, pH 6.5, or 12.5 mM NH_4NO_3, pH 5.5, as the nitrogen source, along with a 10 mM K_2HPO_4-KH_2HPO_4 buffer containing 2 mM $MgSO_4$, 0.1 mM $FeSO_4$·EDTA, 1 mM $CaCl_2$, 50 μM H_3BO_4, 12 μM $MnSO_4·H_2O$, 1 μM $ZnCl_2$, 1 μM $CuSO_4·5H_2O$, 0.2 μM $Na_2MoO_4·2H_2O$, 1 g/L Mes, and 0.5% sucrose. Plants were grown under 24 hr of continuous light and rotated at 80 rpm for 12 days at 24°C. For $(NH_4)_2$ succinate–grown plants, seedlings were washed twice with 10 mM K_2HPO_4-KH_2PO_4 buffer, pH 5.5 or pH 7.0, and resuspended into 13 mL of nutrient solution with 250 μM KNO_3, pH 5.5 or pH 7.0, for uptake studies. For plants grown in NH_4NO_3, seedlings were washed and pretreated with 25 mL of nutrient solution without nitrogen for 3 hr before shifting to the uptake assay solution of 250 μM KNO_3, pH 5.5. Samples of the solution were taken from the flask at different time points, and the amount of nitrate left in the solution was determined by HPLC analysis (Thayer and Huffaker, 1980) using a PARTISIL 10 SAX column (Whatman, Clifton, NJ). All experiments were performed in triplicate.

RNA Gel Blot Analysis

For the determination of the *CHL1* mRNA level in the *chl1* mutants, plants were grown vertically on agarose plates with 12.5 mM $(NH_4)_2$ succinate, pH 6.5, and 0.45% agarose (FMC BioProducts, Rockland, ME) for 18 days, as previously described (Tsay et al., 1993). The plants were then shifted to plates with 25 mM KNO_3 as the nitrogen source at pH 5.5 for 2 hr. To measure the time course of *CHL1* mRNA expression, we grew wild-type and 35S–*CHL1* transgenic plants under the same conditions used for the nitrate uptake assay described above. Total RNA from root tissues was isolated by TRIzol reagent (Gibco BRL, Grand Island, NY). Hybridization was performed at 65°C in 5 × SSC (1 × SSC is 0.15 M NaCl and 0.015 M sodium citrate), 5 × Denhardt's solution (1 × Denhardt's solution is 0.2 g/L Ficoll, 0.2 g/L

polyvinylpyrrolidone, and 0.2 g/L BSA), 0.5% SDS, and 25 μg/mL salmon sperm DNA. Washing conditions were 2 × SSC and 0.1% SDS at 65°C for 40 min and then 0.2 × SSC and 0.1% SDS at 65°C for 40 min.

Chlorate Sensitivity Assay

Vermiculite/perlite mixed at 2:1 ratio was saturated with a pH 5.5 solution of 10 μM chlorate containing 2.5 mM $(NH_4)_2SO_4$, 10 mM $K_2HPO_4{\cdot}KH_2PO_4$, 2 mM $MgSO_4$, 0.1 mM $FeSO_4{\cdot}$EDTA, 1 mM $CaCl_2$, 50 μM KNO_3, and other micronutrients as listed above. Seeds were sown in pots and vernalized at 4°C for 3 days and then grown at 24°C under continuous light. Plants were treated with 10 μM chlorate solution three more times at 3, 6, and 9 days after germination.

Nitrate Uptake Assay in Xenopus Oocytes

Oocytes were isolated and injected with 50 ng of *CHL1* mRNA or *NTL1* mRNA, as previously described (Tsay et al., 1993). Two different procedures were performed for nitrate uptake of oocytes. For lower concentrations of nitrate (12 to 400 μM), batches of five oocytes were incubated in a 200-μL solution containing HNO_3, 230 mM mannitol, 0.3 mM $CaCl_2$, and 10 mM Mes-Tris, pH 5.5 or pH 7.4. Solution samples (100 μL) were removed at 0 and 3 hr, and nitrate left in the solution was analyzed by HPLC. For higher concentrations of nitrate (0.11 to 30 mM), oocytes were incubated in a 500-μL solution of HNO_3, 230 mM mannitol, 0.3 mM $CaCl_2$, and 10 mM Mes-Tris, pH 5.5, for 3 hr and then rinsed five times with water. A batch of five oocytes was subsequently lysed in 200 μL of water by pipetting through a thin pipette tip, followed by filtration with syringe filters (Xpertek, St. Louis, MO). The retained nitrate was analyzed by HPLC.

ACKNOWLEDGMENTS

This work was supported by grants from Academia Sinica and the National Science Council of Taiwan (Grant Nos. NSC-86-2311-B-001-011 and NSC-87-2311-B-001-077). We thank the anonymous reviewer and the coeditor, whose comments and suggestions contributed significantly to the rigor of this paper.

Received December 1, 1998; accepted March 3, 1999.

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NOTE ADDED IN PROOF

Reduced high-affinity nitrate uptake activities of the *chl1* mutant have been independently observed (**Wang, R., Liu, D., and Crawford, N.W.** [1998]. The *Arabidopsis* CHL1 protein plays a major role in high-affinity nitrate uptake. Proc. Natl. Acad. Sci. USA **95,** 15134–15139).